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(54) Title: NOVEL CHEMOKINE RECEPTOR INHIBITION ASSAY

(57) Abstract

Ligands MCP-4, MCP-3, RANTES, MCP-2, MCP-1 or eotaxin, has been identified for the G-protein receptor HGBER32.

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NOVEL CHEMOKINE RECEPTOR INHIBITION ASSAY

This invention relates to the identification of a novel receptor for a human CC-chemokine and its use as a screening tool to identify inhibitors of the receptor, to inhibitors so identified and their use in therapy.

Chemokines are structurally and functionally related 8 to 10 kD polypeptides, involved in the recruitment of white blood cell into areas of inflammation and their subsequent activation (Miller, M.D. and Krangel, M.S. (1992) *Crit. Rev. Immunol.* **12**, 17-46; Baggiolini, M., Dewald, B. and Moser, B.(1994) *Adv. Immunol.* **55**, 97-179). In addition, some chemokines are able to regulate the proliferative potential of hematopoietic progenitor cells, endothelial cells and certain types of transformed cells (Oppenheimer, J.J., Zachariae, C.O.C., Mukaida, N., and Matsushima, K. (1991) *Ann. Rev. Immunol.* **9**, 617-648; Schall, T.J. (1991) *Cytokine* **3**, 165-183). Based on whether the first two cysteine moieties are separated by one amino acid moiety or are adjacent, chemokines belong to the α- or CXC chemokine family (e.g interleukin (IL)-8) or the β- or CC chemokine family (e.g. RANTES and MCP-1). CXC chemokines preferentially attract and affect neutrophils while CC chemokines chemoattract and affect eosinophils, monocytes and T-cells with relative potencies that differ between the different members of this family.

Chemokines express their biological responses through interaction with chemokine receptors (Horuk, R. and Peiper, S.C. (1995) *Exp. Opin. Ther. Patents* **5**, 1185-1200).

Several chemokine receptors have already been cloned, for instance, the following human CXC chemokine receptors:
IL-8A and IL-8B (Holmes, W.E., Lee, J., Kuang, W.J., Rice, G.C. and Wood, W.I. (1991) *Science* **253**, 1278-1280; Murphy, P.M. and Tiffany, H.L. (1991) *Science* **253**, 1280-1283);
as well as the following human CC chemokine receptors:
MIP-1α/RANTES receptor (CC-CKR-1) (Neote, K., Digregorio, D., Mak,J.K., Horuk, R. and Schall, T.J. (1993) *Cell* **72**, 415-425; Gao, B. J-L., Kuhns, D.B., Tiffany, H.L., McDermott, D., Li, X., Francke, U. and Murphy, P.M. (1993) *J. Exp. Med.* **177**, 1421-1427);
MCP-1A and B receptors (CC-CKR-2A and B) (Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2752-2756; Yamagami, S., Tokuda, Y., Ishii, K., Tanaka, T. and Endo, N. (1994) *Biochem. Biophys. Res. Commun.* **202**, 1156-1162);
the eotaxin/RANTES receptor (CC-CKR-3) (Combadiere, C., Ahuja, S.K. and Murphy, P.M. (1995) *J. Biol. Chem.* **270**, 16491-16494; Daugherry, D.L., Siciliano, S.J., DeMartino, J.A., Malkowitz, L., Sirotina, A. and Springer, M.S. (1996) *J.Exp.Med.* **183**, 2349-2354; Kitaura, M.,

Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H.L., Murphy, P.M. and Yoshie, O. (1996) *J. Biol Chem.* **271**, 7725-7730),
the promiscuous receptor on basophils CC-CKR-4 (Power, C.A., Meyer, A., Nemeth, K., Bacon, K.B., Hoogewerf, A.J., Proudfoot, A.E.I. and Wells, T.N.C. (1995) *J. Biol. Chem.* **270**, 19495-19500); and
a new MIP-1α/MIP-1β/RANTES receptor (CC-CKR-5) (Samson, M., Labbe, O., Mollereau, C., Vassart, G. and Parmentier, M. (1996) *Biochemistry* **35**, 3362-3367.

Chemokine receptors belong to the group of 7 transmembrane (7TM) spanning receptors and their signal transduction pathway involves pertussus toxin-sensitive G-protein and a rise in $[Ca^{2+}]_i$. Although details about the molecular events are still incomplete, a complex array of intracellular signals ultimately lead to leucocyte activation and chemotaxis.

Chemokine receptors, like chemokines, are divided into two sub-families, the CXC chemokine receptors (CXCR), and the CC chemokine receptors (CCR), based on their selectivity for either CXC or CC chemokines. Ligand cross-selectivity, that is CXCRs that bind CC chemokines or *vice versa*, is not observed. Chemokine receptors consist of 350-368 amino acids and the sequence identity amongst members of the two receptor sub-families is 36-77 and 46-74%, respectively. Most chemokine receptors recognise more than one chemokine and many chemokines, including IL-8, RANTES, MIP-1α and the MCPs, bind to more than one receptor (Roos *et al*, J Biol Chem, 1997, 272 (28), 17521).

Current research suggests a pathophysiological role for chemokines in diverse inflammatory states arising from viral, bacterial and parasital infection, allergic and asthmatic reactions, atherosclerosis and arthritis and chemokines and their receptors have been recognised as targets for therapeutic agents.

More recently, a further chemokine, originally designated CKβ10 and now tentatively named MCP-4, has been identified (WO 95/31467, Human Genome Sciences, Inc.). This has the amino acid sequence given as SEQ ID NO 1. This chemokine was detected by random sequencing of expressed sequence tags in cDNA libraries.

Bagglioni *et al* (J Exp Med, 1996, 183, 2370-84) have presented the results of some preliminary characterisation of MCP-4. Sequence alignments with known CC-chemokines showed high levels of identity (56-61%) with the three known MCP chemokines and 60% sequence identity to eotaxin. The new chemokine showed marked functional similarities to MCP-3 and eotaxin,

being a chemoattractant of high efficacy for monocytes and T lymphocytes. On these cells, the chemokine is thought to signal through receptors that recognise MCP-1, MCP-3 and RANTES. MCP-4 has since been further described by Berkhout *et al* (J Biol Chem, 1997, 272, 16404). They showed that binding of MCP-4 to monocytes is at least in part due to the CC-CKR-2B (MCP-1) receptor.

There remains unresolved the identification of further potential receptors for MCP-4. WO 95/31467 suggests that a receptor for MCP-4 may be identified by expression cloning or identified from cell membranes or extract preparations that express the receptor, by using labelled MCP-4, according to standard methods well known in the art. There is however no specific disclosure of an MCP-4 receptor, by way of sequence data or pharmacological characterisation.

Receptors may also be identified by searching through sequence databanks for likely candidates, expressing the encoded polypeptide in a suitable cell line and then characterising it. In particular, analysis of expressed sequence tags in cDNA libraries and subsequent obtention of full length sequences has led to the identification of several so-called orphan 7-transmembrane receptors.

Earlier filed application WO 96/39434 (Human Genome Sciences, Inc.) discloses a novel polypetide and DNA (RNA), referred to as 'HGBER32'. This is putatively identified as a G-protein receptor. The protein exhibits the highest degree of homology to MCP-1 with about 40% identity and about 64% similarity. There is however no specific identification of the receptor or of a ligand for this receptor. This hinders the development of screening methods to identify agonists and antagonists of the receptor.

We have now demonstrated that the chemokine MCP-4 binds to the human 7-transmembrane receptor 'HGBER32'. Accordingly, 'HGBER32' is a receptor for MCP-4 and will hereinafter be referred to as the MCP-4 receptor.

Roos *et al* (J Biol Chem, 1997, 272 (28), 17521) have more recently identified a ligand for a receptor named TER-1 which is identical to HGBER32 except for two point mutations. They demonstrated that their orphan chemokine receptor, originally named TER-1, now named CCR8, is a receptor for the CC chemokine I-309.

Identification of the nature of the receptor and of a ligand therefor facilitates the development of screening methods for identifying agonists and antagonists of the receptor.

In a first aspect, the present invention provides for a screening method for identifying antagonists of the MCP-4 receptor which method comprises using the MCP-4 receptor, suitably expressed on the surface of a host cell or in a membrane preparation, or as an isolated protein, in combination with MCP-4.

MCP-4 has the amino acid sequence given in SEQ ID NO:1.

The terms 'MCP-4 activity' and 'biological activity of MCP-4' refers to the metabolic or physiologic function of said MCP-4 including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said MCP-4.

MCP-4 receptor has the amino acid sequence given in SEQ ID NO:2.

The terms 'MCP-4 receptor activity' and 'biological activity of MCP-4 receptor' refer to the metabolic or physiologic function of said MCP-4 receptor including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said MCP-4 receptor.

In screening methods of the present invention, the MCP-4 ligand may be labelled, for instance by a radio label such as ^{125}I or a fluorogenic label, or unlabelled. The degree of antagonism may be determined according to conventional techniques, for instance by measuring the level of binding of MCP-4 or by measuring a change in a functional response or a second messenger system associated with the receptor.

Accordingly, in a further aspect, the present invention provides for the use of MCP-4 in a screening method, in particular, for a method of identifying an antagonist of the MCP-4 receptor which method involves the use of MCP-4 which may be labelled or unlabelled.

Suitably, the screening method comprises the initial steps of expressing and isolating recombinant MCP-4 receptors, and/or their extracellular domains.

Suitable cell lines are well known in the art and include, for instance, cells from mammals, yeast, drosophila and *E. Coli*.

Receptor expression may be transient or stable. Preferably, the expression is stable.

Preferably, a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the MCP-4 receptor, and the cell line then cultured in a culture medium, such that the receptor domain is stably expressed on the outside of the cell.

Suitably, an antagonist is then identified by adding an effective amount of a compound to the culture medium used to propogate the transfected cells expressing the receptor. An effective amount is a concentration sufficient to block the binding of MCP-4 to the receptor. The loss of binding of MCP-4 to the receptor may be assayed using various techniques, using intact cells or in solid phase assays. Thus, for instance, the amount of labelled MCP-4 bound by the receptor in the presence and absence of the test compound may be quantified using standard techniques. Alternatively, an immunoassay may be used to detect MCP-4 binding to its receptor by detecting the immunological reactivity of MCP-4 with anti-MCP-4 antibodies in the presence and absence of the test compound. The immunoassay may for example involve an antibody sandwich or an enzyme linked immunoassay (ELISA). Such methods are well known in the art and described in for instance Methods in Enzymology (1987, vol 154 and 155, Wu and Grossman, and Wu) and Methods in Cell and Molecular Biology (Academic Press, London).

An antagonist may also be identified by measuring the response of a known second messenger system in the presence or absence of test compound. Suitable second messenger systems include cAMP guanylate cyclase, ion channels and phosphoinositide hydrolysis.

An antagonist may also be identified by measuring a functional responses in the presence or absence of test compound. Suitable such responses include cytosolic calcium ion concentrations ($[Ca^{2+}]_i$), effects on ligand-induced chemotaxis and changes in extracellular pH changes caused by receptor activation, as described for instance, in Science, Oct 1989, 246, 181-296.

In addition, screening methods may involve the use of melanophores which are transfected to express the receptor, as described in WO 92/01810; *Xenopus* oocytes in which the receptor is transiently expressed; or systems in which the receptor is linked to a phospholipase C or D.

The identification of HGBER32 as a chemokine receptor, in particular a receptor for MCP-4, allows the easier development of screening methods for identifying agonists of the receptor as it suggests which are the appropriate functional responses to measure.

Accordingly, in a further aspect, the present invention provides for a screening method for identifying agonists of the MCP-4 receptor which method comprises contacting a compound with

MCP-4 receptor expressed on the surface of a host cell or in a membrane preparation and measuring the change in a functional response or a second messenger system associated with the receptor.

Suitably, a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the MCP-4 receptor, the cell line is cultured in a culture medium, such that the receptor domain is stably expressed, an effective amount of a test compound is added to the culture medium, sufficient to activate the receptor, and the change in a functional response is measured.

Binding studies suggest that MCP-3, RANTES, MCP-2, MCP-1 and eotaxin have similar affinities for the MCP-4 receptor. Accordingly, MCP-4 may be replaced by MCP-3, RANTES, MCP-2, MCP-1 or eotaxin in the previously described screening methods for identifying receptor antagonists.

It will be readily appreciated by those skilled in the art that chemokine receptors such as the MCP-4 receptor generally have at least one chemokine ligand. Thus, it is already known that that MCP-4, MCP-3, RANTES, MCP-2, MCP-1 and eotaxin have similar affinities for the MCP-4 receptor. Further ligands may be identified using known ligands and the receptor, using competitive binding studies.

Agonists and/or antagonists may be identified from a variety of sources, for instance, from cells, cell-free preparations, chemical libraries and natural product mixtures. Such agonists and/or antagonists may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of MCP-4; or may be structural or functional mimetics of the polypeptide of the present invention. See Coligan *et al.*, *Current Protocols in Immunology* 1(2):Chapter 5 (1991). Examples of potential MCP-4 receptor antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, substrates, receptors, enzymes, etc., as the case may be, of the MCP-4 receptor, e.g., a fragment of the ligands, substrates, receptors, enzymes, etc.; or small molecules which bind to the MCP-4 receptor but do not elicit a response, so that the activity of the receptor is prevented.

Compounds identified using the screen will be of use in therapy. Accordingly, in a further aspect, the present invention provides a compound identified as an agonist or an antagonist of the MCP-4 receptor for use in therapy.

Compounds thus identified may be used for treating any disease state associated with the MCP-4 receptor, for instance inflammatory states arising from viral, bacterial and parasital infection, allergic and asthmatic reactions, atherosclerosis and arthritis.

Accordingly, in a further aspect, this invention provides a method of treating an abnormal condition related to an excess of MCP-4 receptor activity and/or a ligand thereof which comprises administering to a patient in need thereof an antagonist as hereinbefore described in an amount effective to block binding of ligands to the receptor, or by inhibiting a second signal, and thereby alleviating the abnormal conditions.

This invention also provides a method of treating an abnormal condition related to an under-expression of MCP-4 receptor activity and/or a ligand thereof which comprises administering to a patient in need thereof a therapeutically effective amount of an agonist compound which activates the receptor as hereinbefore described and thereby alleviate the abnormal conditions.

Identification of a ligand for the MCP-4 receptor, such as MCP-4, or MCP-3, RANTES, MCP-2, MCP-1 or eotaxin, allows for the effective identification of polyclonal or monoclonal antibodies raised against the MCP-4 receptor which are neutralising antibodies. Such neutralising antibodies are of use in therapy, in comparison to non-neutralising antibodies which are ineffective. Accordingly, in a further aspect, the present invention provides for the use of neutralising antibodies raised against the MCP-4 receptor in therapy.

Such antibodies may be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain or humanised antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures well known in the art may be used for the production of such antibodies.

Antibodies generated against the MCP-4 receptor may be obtained by direct injection of the isolated receptor into an animal or by administering the receptor to an animal, preferably a non-human. The antibody so obtained willl then bind the receptor. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures may be used, for instance the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the humanB-cell hybridoma technique (Kozbor et al, 1985, Immunology Today, 4:72) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, in Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, 1975: 77-96). Techniques described for the production of single chain antibodies in US 4,946,778 can be

adapted to produce single chain antibodies to immunogenic polypeptides. In addition, transgenic mice may be used to express humanised antibodies to immunogenic polypeptides.

Compounds, including antibodies, for use in such methods of treatment will normally provided in pharmaceutical compositions. Accordingly, in a further aspect, the present invention provides for a pharmaceutical composition comprising a compound identified as an inhibitor or an activator of the MCP-4 receptor and a pharmaceutically acceptable excipient or carrier.

Compounds which are active when given orally can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and, lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilised and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises a compound of formula (I) or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

Preferably the composition is in unit dose form such as a tablet or capsule.

Each dosage unit for oral administration contains preferably from 1 to 250 mg (and for parenteral administration contains preferably from 0.1 to 25 mg) of an inhibitor of the invention.

The daily dosage regimen for an adult patient may be, for example, an oral dose of between 1 mg and 500 mg, preferably between 1 mg and 250 mg, or an intravenous, subcutaneous, or intramuscular dose of between 0.1 mg and 100 mg, preferably between 0.1 mg and 25 mg, of the compound of the formula (I) or a pharmaceutically acceptable salt thereof calculated as the free base, the compound being administered 1 to 4 times per day. Suitably the compounds will be administered for a period of continuous therapy.

Identification of a ligand for the MCP-4 receptor provides a means of developing a diagnostic assay for measuring levels of MCP-4 in a patient. Accordingly, in a further aspect, the present invention provides for a diagnostic assay for detecting diseases or susceptibility to diseases related to abnormal activity of the MCP-4 receptor. Assays used to detect levels of MCP-4, MCP-3, RANTES, MCP-2, MCP-1 or eotaxin in a sample derived from a patient are well known in the art and include radioimmnuoassays, Western blot analysis and ELISA assays. The invention also provides a diagnostic kit comprising MCP-4, MCP-3, RANTES, MCP-2, MCP-1 or eotaxin.

The invention is further described in the following examples which are intended to illustrate the invention without limiting its scope. In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

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All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

Examples

1. Transient and stable expression of MCP-4 receptor in mammalian cell lines

In order to maximize receptor expression, all 5' and 3' untranslated regions (UTRs) were removed from the receptor cDNA (SEQ ID NO 3) prior to insertion into a pCDN expression vector. Since PCR was used to trim the cDNA, the DNA sequences were routinely confirmed prior to expression.

COS cells

Initially, transient transfection of the chemokine receptor in COS cells was used using the dextran sulfate method. Briefly, 1 x 10^7 COS cells were grown in 245x245-mm tissue culture plates for 24 h to 50-70% confuency. The cells were washed with PBS and then tranfected with 100 ug of the chemokine receptor cDNA in DMEM media containing 10% Neuserum 1% glutamine, DE dextran and chloroquine media and incubated for 3 hr. The cells were shocked with 10% DMSO, washed and incubated for three days in DMEM medium containing 10% fetal bovine serum, 1% glutamine.

For stable expression, HEK293 or adherent dhfr-CHO cells were transfected with receptor cDNA by lipofection and selected in the presence of 400 ug/ml G418. After 3 weeks of selection, individual clones were picked and expanded for further analysis. HEK293 and CHO cell clones transfected with pCDN vector alone serve as negative controls.

CHO cells

A fragment containg the entire human receptor cDNA coding sequence was subcloned into the mammalian expression vector pcDN which contains both the G418 resistance and dihydrofolate reductase genes. CHO cells were transfected with the construct using electroporation and plated onto 96 well plates containing F-12 media (GIBCO) supplement with 5% fetal calf serum. After 48 h, the cells were grown in the same media containing neomycin (400 ug/ml) for 2 weeks. Surviving cells were assayed for the production of MCP 4 receptor.

HEK293 cells

500,000 cells were plated onto 100 mm plates in feeding media (EMEM) supplemented with 20 mM L-glutamine 10% fetal bovine serum (FBS) and incubated O/N at 37 °C. The DNA (10ug) in 10 ul of TE buffer and 2 ul of DNA was diluted to 100 ul of serum free EMEM and add 5 ul of lipofectamine to 100 ul of EMEM and incubated at 30 min at room temperature. The cells were washed with PBS and 0.8 ml were added to the cells followed by the DNA/lipofectamine mixture.. The cells were washed with PBS after 5hr and then fresh EMEM serum free media was

added. After 48 hr the cells were trypsinised, diluted and plated in G-418 media. Surviving colonies were grown and tested for the expression of MCP 4 receptor

Expression of recombinant receptor in COS 7 cells and cloning and expression using a Baculovirus expression system is described as Examples 1 & 2 of WO96/39434.

2. Ligand binding studies with receptor

HGBER32 expressing CHO cells were incubated at 37°C with ^{125}I-MCP-4 (0.17 nM) for 15 minutes and indicated chemokines in MR1-3 media, 0.2 % BSA and 0.1 % azide. Cells were collected onto PEI treated GF/C filter plates, filter plates were washed with ice-cold 20 mM HEPES/0.5 M NaCl., pH 7.4 and counted. The results are shown in Figure I.
IC50 values were 0.23 nM for MCP-3, 0.46 nM for RANTES, 0.49 nM for MCP-4, 0.56 nM for MCP-2, 2.9 nM for MCP-1 and 3.3 nM for Eotaxin. I-309 and MIP-1a did not show any competition.

Conclusions
HGBER-32 is a receptor for MCP-4, MCP-3, RANTES, MCP-2, MCP-1 and eotaxin but not for I-309 and MIP-1a.

3. mRNA expression in Different Cell Types

PolyA RNA was isolated from various cell types using guanidinum thiocyanate acid-phenol method. Poly A RNA was isolated using oligo dT column. RNA dot blot analysis was performed with a template manifold apparatus (Schleicher & Schuell, Keene, NH) to assure uniform dot size. Poly A RNA was applied using 0.5 ug of RNA. The RNA samples were denatured by adjusting them to 1M formaldehyde and heating them to 55°C for 15 min. The samples were diluted into 20 volumes of 3 M NaCl containing 0.3 M trisodium citrate and applied to nitrocellulose filters under a gentle vacuum. The filters were washed with additional diluted, baked at 80°C for 2h and then hybridized under high strengency in 50% formamide, 5 X SSPE, 5 X Denhardt's reagent, 0.1% SDS, and 100ug/ml yeast tRNA. The blots was washed with 0.1 X SSC, 0.1% SDS at 50°C and exposed to X-ray film for 48h at -70°C. Quantitation of the dots was performed using Phospho-Imaging analysis.

Analysis of mRNA from freshly isolated human peripheral blood monocytes, THP-1 cells, MonoMac-6 cells, human T-lymphocytes all showed expression of the MCP-4 receptor while the signal with RNA from neutrophils, smooth muscle cells and endothelial cells was not greater than seen with the blank of yeast RNA.

4. Cloning and expression of recombinant human MCP-4

This is described by Berkhout *et al*, J Biol Chem, 1997, 272, 16404-16415.

5. Membrane Preparation and High Throughput Screen for the MCP-4 Receptor

For the discovery of antagonists and agonists of the MCP-4 receptor, a MCP-4 binding competition assay is most useful. As source of the MCP-4 receptor, CHO or HEK 293 cells, stably transfected with the MCP-4 receptor, could be used although other cells transfected with the MCP-4 receptor or cells that naturally show a high level expression of the MCP-4 receptor could also be employed. Typically the culture of cells expressing the MCP-4 receptor (see example 1) is scaled up to 30L and cells are recovered by centrifugation at 600 x g for 10 min. The cell pellet is then frozen in liquid nitrogen. Pellets usually contain around 10^9 cells. For membrane isolation, pellets are freeze/ thawed 3 times. They are then resuspended in ice cold 10mM Tris (pH 7.5), 1 mM EDTA (sodium salt) (40 mls/1e8 cells) and homogenized using a Dounce (glass/glass) homogenizer (20-25 strokes), followed by a Polytron suspension with 3--10 sec pulses on a 3/4 setting (Brinkman tissue homogenizer). This suspension is centrifuged at 300 x g for 10 min. Pellet is discarded and the supernatent fraction is centrifuged at 40,000 x g (Sorvall SS-34: 18,000 rpm) for 30 min. at 4 °C. Pellet is resuspended in homogenizing buffer using the polytron and washed one time. The pellet is resuspended in assay buffer (50 mM Tris pH 7.5) at a concentration of 1 - 4 mg protein/ml.

Membranes obtained this way are suitable for the set-up of a high throughput MCP-4 binding competition assay to search for compounds that interfere in the ligand-receptor interaction. The total binding of MCP-4 to these membranes is first tested to be linear with the amount of membranes used. The time period to reach equilibrium binding at a suitable temperature is also established and is in our experience about 1 h at a temperature of 20 °C. For the screening assay typically 25 µg of membrane protein per well is used in a total volume of 100 µl buffer containing 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5 % Bovine serum albumin (western blot quality), pH 7.4. The concentration of ^{125}I-MCP-4 is typically 1-2 nM and 75,000 cpm /well. Specific binding of ^{125}I-labeled MCP-4 should be displaced completely by unlabeled MCP-4 at concentrations of 100 nM or more. The compounds to be tested are typically disolved and added in DMSO and final concentrations of DMSO in the assay are 1 % or less. After incubation the contents of the wells are harvested on a polyethyleneimine-treated GF/C filter using a 96 well plate cell harvester and the filters is washed four times with typically 1 ml icecold wash buffer containing 20 mM HEPES 0.5M NaCl pH 7.4. To determine any antagonists of ^{125}I-MCP-4 binding, the filters are counted on a gamma counter.

Active compounds are further evaluated for their effect on ligand (MCP-4 or MCP-3) induced transient increases in intracellular calcium concentration. This assay is also able to distinguish whether compounds identified by the membrane binding assay are antagonists or agonists.

For measurements of cytosolic Ca^{2+} concentrations, appropriate cell lines transfected with the MCP-4 receptor should be used. Appropriate cells include CHO cells and HEK 293 cells, if required co-transfected with appropriate G -coupling proteins. For the intracellular calcium concentration measurements cells are incubated with 0.5 μM FURA-2AM for 30 minutes at 37°C in HEPES-buffered saline (145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 10mM HEPES and 10mM glucose), pH 7.4 at 37°C, supplemented with 1% albumin (w/v) and 1mM $CaCl_2$. After loading with FURA-2 the cells are centrifuged for 5 minutes at 300*g*, then resuspended in buffer containing no added albumin, to a cell density of 1.5×10^6 cells /ml, and kept at room temperature until use. Typically, this protocol results in a cytosolic FURA-2 concentration of approx. 100μM. Serial dilutions of chemokines in PBS + 0.1% albumin (w/v) - sterile-filtered - are added to aliquots (0.7ml) of cell suspension. FURA-2 fluorescence is measured at 37°C in a single excitation, single emission (500nm) wavelength Perkin Elmer LS5 fluorimeter. $[Ca^{2+}]_i$ are calculated from changes in fluorescence measured at a single excitation wavelength of 340nm, as described by Grynkiewicz,G., Poenie, M.,& Tsien, R.Y. (1985) *J. Biol. Chem.* **260**, 3440-3450. Compound are normally added to the cells in DMSO solutions (final concentration less than 0.2%) and if compounds have agonist properties an effect on $[Ca^{2+}]_i$ is observed. Receptor function antagonism is evident if compounds are able depress the signal induced by the subsequent addition of MCP-4 or MCP-3 at a concentration shown to give a 75% maximal calcium signal in the absence of any compound.

The effects of compounds on MCP-4 or MCP-3 binding and response in physiologically relevant cells expressing the MCP-4 receptor are normally tested in freshly isolated peripheral blood monocytes or peripheral blood lymphocytes.

6.1 Monocyte isolation

Human peripheral blood monocytes are prepared from the blood of normal healthy volunteers, essentially as described by Boyum (*Methods in Enzymology* (Academic Press, New York and London) **108**, 88-102). Blood is collected into anticoagulant (one part 50mM EDTA, pH 7.4, to nine parts blood), then centrifuged for 5 minutes at 600*g*. The upper layer of platelet-rich plasma is removed and centrifuged for 15 minutes at 900*g*, to pellet the platelets. The upper layer of platelet-poor plasma is removed and added back to the packed red cells; the pelleted platelets are discarded. Dextran T500 is added (10 volumes EDTA blood to one volume 6% (w/v) dextran in

0.9% (w/v) NaCl) and the erythrocytes are allowed to sediment at unit gravity for 30 minutes. The resultant leukocyte-rich plasma is removed and centrifuged for 5 minutes at 400*g*. The cell pellet is resuspended in 5ml of the supernatant, and the suspension is underlayered with 3ml NycoPrep, then centrifuged for 15 minutes at 600*g*. The mononuclear layer at the interface between the plasma and the NycoPrep isremoved and washed through PBS by centrifugation for 5 minutes at 400*g*. The mononuclear layer typically contained ≥ 80% monocytes, determined by staining cytocentrifuge preparations for non-specific esterase using α-naphthyl-butyrate. Cell viability (typically >95%) is assessed as the ability to exclude trypan blue.

6.2 Chemokine binding and binding displacement studies

Monocytes are suspended in RPMI 1640 medium without bicarbonate containing 0.2% BSA and 0.1 % azide. ^{125}I-labeled chemokines (MCP-1 or RANTES, about $2x10^4$ cpm) is incubated with $1\text{-}2x10^6$ cells in the presence or absence of increasing concentrations of unlabeled chemokines (MCP-1, MCP-3, MCP-4, RANTES or MIP-1α) for 15 min at 37°C in a 96 well plate in a final volume of 0.2 ml. After the incubation, 0.5 ml of ice-cold wash buffer (20mM Tris/0.5 M NaCl, pH 7.4) is added and cells collected onto a polyethyleneimine-treated Whatman GF/C filter using a Brandell Cell Harvester. Filters are washed with 4 ml cold wash buffer and the radioactivity bound to the filters counted in a gamma counter. For competition studies, the IC_{50} is calculated with a curve fitting programme (GraFit, Erithacus Software, London), using a four parameter logistic:

$$cpm_{bound} = cpm_{max}/(1 + ([L]/IC_{50})^s) + cpm_{ns}$$

where cpm_{max} represent the binding without competitor, L is the competitor concentration, cpm_{ns} the non-specific binding and s the slope factor

The cpm_{bound} is corrected for "no cell" controls. To obtain the K_d and capacity of binding for individual chemokines, specific binding data from homologous displacement experiments are fitted into a single site ligand binding equation using the GraFit best fit programme

6.3 Measurement of $[Ca^{2+}]_i$

Cytosolic Ca^{2+} concentrations in monocytes may be measured using procedures essentially as decribed above (section 5).

6.4 Monocyte chemotaxis assay

Cell migration is evaluated using a 48-well modified Boyden microchemotaxis chamber (Neuroprobe, Cabin John, USA). 25µl aliquots of chemokines, diluted in RPMI 1640 containing 0.1% BSA (w/v) are placed in the wells of the lower compartment, and 50µl aliquots of monocyte suspension in RPMI 1640 (1.5×10^6 cells/ml) placed in the upper wells of the chamber; the two components being separated by a polycarbonate filter with 5µm pore size. The

chamber is incubated at 37°C in humidified air containing 5% CO_2, for 60 minutes. After incubation, the filter sheet is removed, and the non-migrated cells scraped from its upper surface. The filter is stained with Diff-Quik, and the numbers of migrated cells counted (5 fields per well), using a 40x objective and 10x ocular containing a $10mm^2$ counting grid.

Claims

1. A screening method for identifying antagonists of the MCP-4 receptor which method comprises using the MCP-4 receptor in combination with MCP-4.

2. A method as claimed in claim 1 in which the MCP-4 receptor is expressed on the surface of a host cell or in a membrane preparation.

3. A method as claimed in claim 1 in which the MCP-4 receptor is used in the form of the isolated protein.

4. A method as claimed in claim 1 which comprises the initial steps of expressing and isolating recombinant MCP-4 receptors, and/or their extracellular domains.

5. A method as claimed in claim 4 in which a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the MCP-4 receptor, and the cell line then cultured in a culture medium, such that the receptor domain is stably expressed.

6. A method as claimed in claim 5 in which an antagonist is identified by adding an effective amount of a compound to the culture medium used to propagate the transfected cells expressing the receptor and then measuring the loss of binding of MCP-4.

7. A method as claimed in claim 6 in which the MCP-4 is labelled using a radiolabel or a fluorogenic label and the amount of labelled MCP-4 bound by the receptor is measured in the presence and absence of the test compound.

8. A method as claimed in claim 6 in which the response of a known second messenger system is measured in the presence or absence of test compound.

9. A method as claimed in claim 6 in which the level of a functional response is measured in the presence or absence of test compound.

10. A method as claimed in claim 6 which involve the the use of melanophores which are transfected to express the receptor; *Xenopus* oocytes in which the receptor is transiently expressed; or systems in which the receptor is linked to a phospholipase C or D.

11. A method as claimed in any of the preceding claims in which MCP-4 is replaced by MCP-3, RANTES, MCP-2, MCP-1 or eotaxin.

12. A screening method for identifying agonists of the MCP-4 receptor which method comprises contacting a compound with MCP-4 receptor and measuring the change in a functional response or a second messenger system associated with the receptor.

13. A method as claimed in claim 12 in which the MCP-4 receptor is expressed on the surface of a host cell or in a membrane preparation

14. The use of MCP-4 in a screening method for identifying an antagonist of the MCP-4 receptor which method involves the use of MCP-4 which may be labelled or unlabelled.

15. The use of MCP-3, RANTES, MCP-2, MCP-1 or eotaxin in a screening method for identifying an antagonist of the MCP-4 receptor which method involves the use of MCP-3, RANTES, MCP-2, MCP-1 or eotaxin, respectively, which may be labelled or unlabelled.

16. A compound identified by any one of the screening methods defined in any one of the preceding claims for use in therapy.

17. A method of treating an abnormal condition related to an excess of MCP-4 receptor activity and/or an excess of a ligand thereof which comprises administering to a patient in need thereof a therapeutically effective amount of an antagonist of the receptor identified using any one of the screening methods defined in any one of the claims 1 to 13.

18. A method of treating an abnormal condition related to an under-expression of MCP-4 receptor activity which comprises administering to a patient in need thereof a therapeutically effective amount of an agonist compound using the screening method defined in claim 12.

19. A pharmaceutical composition comprising a compound identified by any one of the screening methods defined in any one claims 1 to 14 and a pharmaceutically acceptable excipient or carrier.

20. A method of diagnosising susceptibility to disease states associated with abnormal expression of the MCP-4 receptor which method comprises measuring the level of MCP-4 and/or MCP-3 in a sample taken from a patient.

21. The use of MCP-4, MCP-3, RANTES, MCP-2, MCP-1 or eotaxin to identify neutralising antibodies to the MCP-4 receptor.

22. Antibodies as defined in claim 22 for use in therapy.

23. The use of MCP-4, MCP-3, RANTES, MCP-2, MCP-1 or eotaxin and the MCP-4 receptor to identify further chemokine ligands for the receptor, using a competitive binding assay and labelled MCP-4 and/or MCP-3.

Figure I

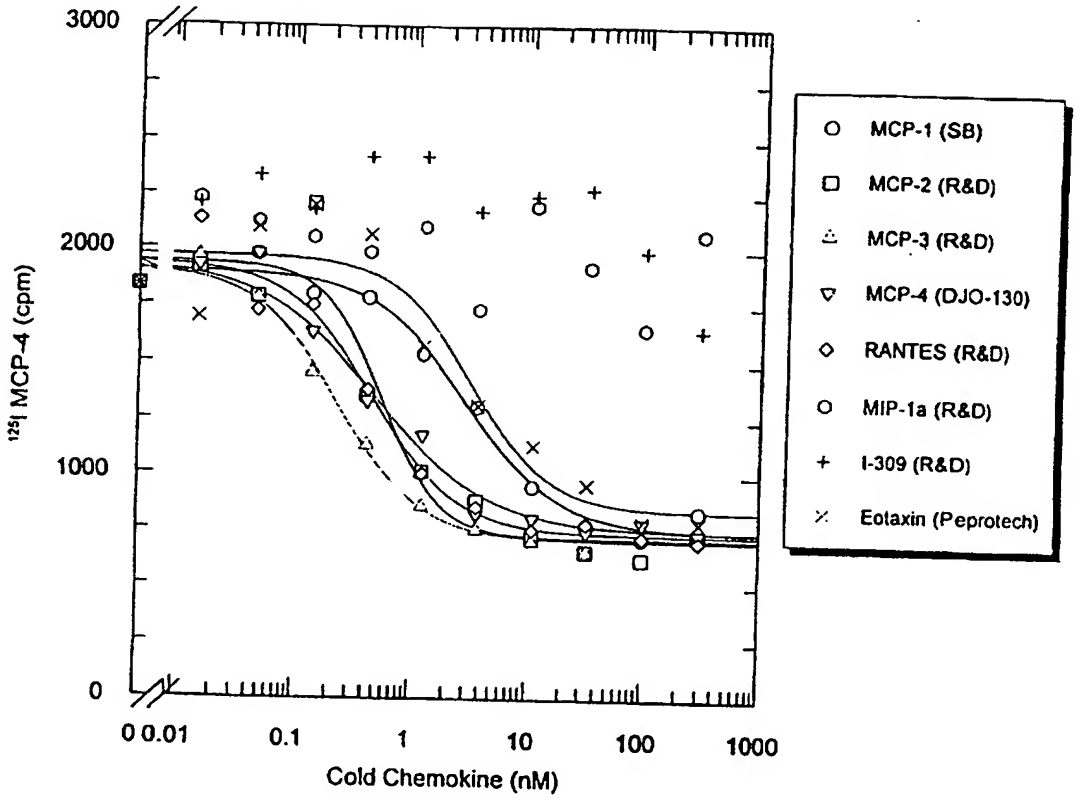
3000
2000
1000
0
^{125}I MCP-4 (cpm)
0 0.01
0.1
1
10
100
1000
Cold Chemokine (nM)
MCP-1 (SB)
MCP-2 (R&D)
MCP-3 (R&D)
MCP-4 (DJO-130)
RANTES (R&D)
MIP-1a (R&D)
I-309 (R&D)
Eotaxin (Peprotech)

Figure II

SEQ ID NO 1

QPDALNVPSTCCFTFSSKKISLQRLKSYVITTSRCPQKAVIFRTKLGKEICADPKEKWVQ NYMKHLGRKAHTLKT

SEQ ID NO 2

```
  1  MDYTLDLSVT TVTDYYYPDI FSSPCDAELI QTNGKLLLAV FYCLLFVFSL
 51  LGNSLVILVL EVCKKLRSIT DVYLLNLALS DLLFVFSFPF QTYYLLDQWV
101  FGTVMCKVVS GFYYIGFYSS MFFITLMSVD RYLAVVHAVY ALKVRTIRMG
151  TTLCLAVWLT AIMATIPLLV FYQVASEDGV LQCYSFYNQQ TLKWKIFTNF
201  KMNILGLLIP FTIFMFCYIK ILHQLKRCQN HNKTKAIRLV LIVVIASLLF
251  WVPFNVVLFL TSLHSMHILD GCSISQQLTY ATHVTEIISF THCCVNPVIY
301  AFVGEKFKKH LSEIFQKSCS QIFNYLGRQM PRESCEKSSS CQQHSSRSSN
351  VDYIL*
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SEQ ID NO 3

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 51  CCCTGATATC TTCTCAAGCC CCTGTGATGC GGAACTTATT CAGACAAATG
101  GCAAGTTGCT CCTTGCTGTC TTTTATTGCC TCCTGTTTGT ATTCAGTCTT
151  CTGGGAAACA GCCTGGTCAT CCTGGTCCTT GAGGTCTGCA AGAAGCTGAG
201  GAGCATCACA GATGTATACC TCTTGAACCT GGCCCTGTCT GACCTGCTTT
251  TTGTCTTCTC CTTCCCCTTT CAGACCTACT ATCTGCTGGA CCAGTGGGTG
301  TTTGGGACTG TAATGTGCAA AGTGGTGTCT GGCTTTTATT ACATTGGCTT
351  CTACAGCAGC ATGTTTTTCA TCACCCTCAT GAGTGTGGAC AGGTACCTGG
401  CTGTTGTCCA TGCCGTGTAT GCCCTAAAGG TGAGGACGAT CAGGATGGGC
451  ACAACGCTGT GCCTGGCAGT ATGGCTAACC GCCATTATGG CTACCATCCC
501  ATTGCTAGTG TTTTACCAAG TGGCCTCTGA AGATGGTGTT CTACAGTGTT
551  ATTCATTTTA CAATCAACAG ACTTTGAAGT GGAAGATCTT CACCAACTTC
601  AAAATGAACA TTTTAGGCTT GTTGATCCCA TTCACCATCT TTATGTTCTG
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 651  CTACATTAAA ATCCTGCACC AGCTGAAGAG GTGTCAAAAC CACAACAAGA
 701  CCAAGGCCAT CAGGTTGGTG CTCATTGTGG TCATTGCATC TTTACTTTTC
 751  TGGGTCCCAT TCAACGTGGT TCTTTTCCTC ACTTCCTTGC ACAGTATGCA
 801  CATCTTGGAT GGATGTAGTA TAAGCCAACA GCTGACTTAT GCCACCCATG
 851  TCACAGAAAT CATTTCCTTT ACTCACTGCT GTGTGAACCC TGTTATCTAT
 901  GCTTTTGTTG GGGAGAAGTT CAAGAAACAC CTCTCAGAAA TATTTCAGAA
 951  AAGTTGCAGC CAAATCTTCA ACTACCTAGG AAGACAAATG CCTAGGGAGA
1001  GCTGTGAAAA GTCATCATCC TGCCAGCAGC ACTCCTCCCG TTCCTCCAAC
1051  GTAGACTACA TTTTGTGA
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02313

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 G01N33/566 C07K14/71 C07K14/72 C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 31467 A (HUMAN GENOME SCIENCES INCORPORATED) 23 November 1995 cited in the application see the whole document	1-23
A,P	WO 96 39434 A (HUMAN GENOME SCIENCES INCORPORATED) 12 December 1996 cited in the application see the whole document	1-23
A,P	WO 96 40762 A (HUMAN GENOME SCIENCES INCORPORATED) 19 December 1996 see the whole document	1-23
E	WO 97 31098 A (HUMAN GENOME SCIENCES INCORPORATED) 28 August 1997 see examples 4-12	1-23

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
4 December 1997	18/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02313

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531467 A	23-11-95	AU 6913094 A	05-12-95
		EP 0767796 A	16-04-97
WO 9639434 A	12-12-96	AU 2766395 A	24-12-96
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WO 9731098 A	28-08-97	AU 5355996 A	10-09-97